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# Development of Water and Soil Treatment Technology Based on the Utilization of a White-Rot, Wood Rotting Fungus

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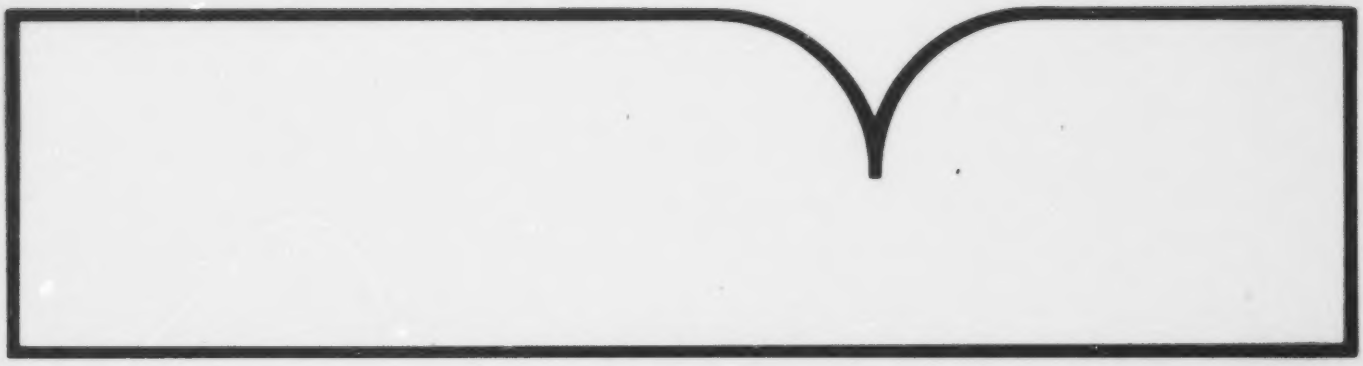

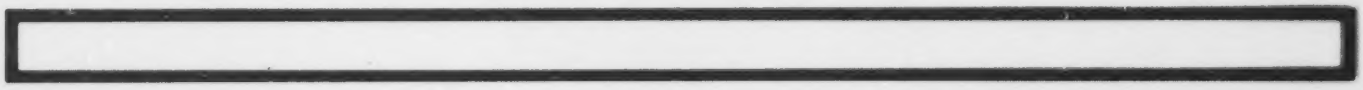

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# THE DEVELOPMENT OF WATER AND SOIL TREATMENT TECHNOLOGY BASED ON THE UTILIZATION OF A WHITE-ROT, WOOD ROTTING FUNGUS

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16. ABSTRACT

The wood rotting fungus, Phanerochaete chrysosporium has been selected as a candidate species to be used as a degrader of hazardous waste organic consistuents found in liquids and soils. The selection of this species is attributable to its rapid growth, its ability to degrade lignin rapidly, its ability to asexually multiply, and its high temperature optimum. Based on the fungus' ability to degrade lignin several investigators speculated that the fungus should be able to degrade aromatic organic constituents found in hazardous waste. Early studies with the polychlorinated biphenyl mixture Arochlor 1254, DDT, Lindane and other chlorinated contaminants indicated that the fungus may have exceptional degradative abilities. The lignin degrading ability of the fungus is a secondary metabolic cycle that is controlled by the absence of certain nutrients.

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# THE DEVELOPMENT OF WATER AND SOIL TREATMENT TECHNOLOGY BASED ON THE UTILIZATION OF A WHITE-ROT, WOOD ROTTING FUNGUS

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## INTRODUCTION

The detoxification of hazardous waste is becoming an important objective in the reduction of risk associated with such waste materials. Detoxification refers to the conversion of a toxicant to innocuous metabolites; it does not necessarily mean that the target substrate has been mineralized. Mineralization is the conversion of toxicant substrates to carbon dioxide and inorganic products. The potential of biological means to detoxify hazardous waste is beginning to be realized through recent technology developments. Greater environmental compatibility and potentially lower cost are significant inducements permitting biological treatment technology to assume a more competitive status for site cleanup. In spite of these very promising aspects, biological detoxification must be recognized as a fledgling technology having excellent credentials in the areas of municipal and industrial wastes but underdeveloped for the treatment of mixtures of more toxic and persistent chemicals found as components of hazardous waste sites.

A major hazardous waste problem confronting authorities in the United States is the waste associated with the wood treatment industry. Depending on the age of a facility, the accumulated waste can be derived from a mixture of at least three technologies. Historically, creosote treatment was followed by pentachlorophenol which was replaced with copper chromated arsenite. Each of these technologies present its special conditions for cleanup. Creosote, derived from coal tar produc-

tion, usually contained a host of compounds ranging from straight aromatic compounds to polyaromatic species including smaller quantities of aromatic nitrogen bases and an array of phenolic compounds. Pentachlorophenol is a potent fungicide leading to its selection for wood preservation technology. The analysis of wastes derived from the pentachlorophenol technology have identified other potential toxic components. For our current development efforts, we have narrowly focussed on a significant portion of the waste including the major contributors that are polycyclic aromatic compounds and phenols. It is necessary to limit the scope to permit the treatment objective to be achieveable.

## BACTERIA VERSUS FUNGI

Microorganisms (both bacteria and fungi) are known to possess a variety of detoxification skills, associated with the utilization of new sources of energy (for instance xenobiotics) and the need to survive (1). Many bacteria can accomplish simple transformations on organic substrates but often fail to complete the conversion of toxicant substrate to carbon dioxide. The use of bacterial communities recognizes these deficiencies through the combined use of many species where the abilities of one species supplants the inadequacies of another. Since the collective action of these communities is important to treatment success, it is important to protect them from environmental effects that may adversely affect the communities.

Fungi have not been investigated to any extent for use as degraders of waste materials until recently (2). Sewage treatment operations steered clear of filamentous fungi due to processing problems and the possibility that such fungi may be pathogenic. Exceptions to these generalization do exist. Some sixty years ago Falck and Haag reported the ability of wood rotting fungi to degrade phenols (3). A wood rotting basidiomycetes, Trametes versicolor, was studied, twenty-five years ago, spectrophotometrically in an attempt to quantify its degrading ability (4).

A ligninolytic fungus, Phanerochaete chrysosporium characterized by fast growth and easy reproductive cycles degrades an increasing list of hazardous waste consistuents under laboratory conditions. This ability to degrade hazardous pollutants appears to correlate well with the fungus' ability to degrade lignin, a complex natural polymer composed of phenylpropane units that is resistant to decay by most organisms.

Some of the more common substructures of lignin, 1,2-aryl diethers, alkyl sidechains, and connected aryl systems, resemble the chemical structure of many persistent organic compounds

contaminating the environment. The remarkable similarity in structures offered a connection for several investigators to pursue application of a white rot fungus, P. chrysosporium to the biodegradation of hazardous waste constituents (5). The early findings of Aust (6) and Eaton (7), have propelled the area of application of the white rot fungus, P. chrysosporium to the detoxification of hazardous waste constituents significantly. Comparison of enzyme activity between the extracellular enzymes of P. chrysosporium and other peroxidases point out that the fungus' extracellular enzymes are among the most powerful biological oxidation systems known.

## TYPES OF WOOD ROTTING FUNGI

There are some 1600 different wood rotting fungi known. These organisms are divided into three main categories: soft rot, white and brown rotting fungi. The identification of white or brown does not refer to the growing appearance of the fungi but rather to the residue left after the fungus has infested a suitable host. The brown coloration characteristic of a brown rot, wood rotting fungus is attributed to incompletely degraded lignin and can be contrasted with the white rot fungi that have more complete capabilities to degrade lignin.

P. chrysosporium is a filamentous, white, wood rotting fungus and has been typed to be a member of the Hymenomycetes subclass of Basidiomycetes (8). Fungi are eukaryotic, ie. they possess a nuclear membrane and as microorganisms are considered to be plantlike without chlorophyll having no photosynthetic abilities (9).

## WOOD ROOTING FUNGI AS CARBON STRUCTURE DEGRADERS

White rot fungi are primary wood degraders in nature (10). The naturally occurring polymers of cellulose and lignin are degraded by these fungi forming the major sources of carbon to assist fungal growth. Of the two general polymers, lignin, a structural component of wood, is by far the more difficult to degrade due to its composition as a heteropolymer formed from the cross linking of three precursor cinnamyl alcohols (11). Of necessity, the fungus must be able to switch its ability degrade these various polymers as the concentration of polymer varies with the composition of the wood. This ability for P. chrysosporium is controlled by the absence of certain nutrients. Nitrogen deficiency is generally used to induce this secondary metabolic cycle of lignin utilization.

## THE IMPORTANCE OF EXTRACELLULAR ENZYMES

The enzyme systems responsible for the initial attack on lignin require unusual abilities due to the complexity and resistance of the lignin structure. The 600-1000 k-dalton size range for lignin is far too large to enter the cells of microorganisms by known transport systems. An enzyme system permitting the microorganism to overcome this limitation must be extracellular, non-specific (due to the heterogeneity and large molecular weight of the substrate), and not susceptible to protease destruction. Analogies with other biopolymers degrading extracellular, non-specific (due to the heterogeneity and large systems fail since these other systems are hydrolytic and specific (11).

Lignin degradation is viewed as being accomplished in two distinct compartments: extracellular and intracellular. The extracellular lignin degrading enzymes serve to fragment lignin into pieces that can be assimilated by the fungus. This model stresses the importance of the individual enzyme's activity and function. Little is known of the intracellular enzyme components that complete the conversion of the lignin fragments into carbon dioxide.

Among the more important reactions in lignin breakdown by P. chrysosporium are the cleavage of lignin alkyl sidechains, ring demethylation, and ring cleavage. The alkyl sidechain cleavage is catalyzed by a hemoprotein ligninase. Hydrogen peroxide is consumed in this reaction with corresponding changes in the enzyme absorption spectrum during catalysis attributable to resting states and transient intermediates indicating a peroxidative mechanism. Stoichiometries of product formation as well as hydrogen peroxide and oxygen uptake are consistent with a radical pathway (12). These results established the one-electron oxidative mechanism as the primary extracellular oxidative pathway for P. chrysosporium.

## DEGRADATION STUDIES OF WASTE CONSTITUENTS

Radiorespirometric studies of the degradation of [U-$^{14}$C] pentachlorophenol in aqueous media indicated that the substrate was rapidly converted to carbon dioxide. Extracellular enzyme studies showed that pentachlorophenol is converted to the 1,4-tetrachlorobenzoquinone by the fungus (13). The quinone is difficult to quantify due to its propensity to form charge transfer complexes with cellular materials. Further elucidation of the metabolic pathway is in progress. Several aromatic hydrocarbons, benzo[a]anthracene, pyrene, anthracene, benzo[a]pyrene and pyrelene, (potential constituents of

creosote), were converted to carbon dioxide by the fungus in liquid culture (14,15). This latter finding serves to differentiate the fungus from bacterial species since few bacteria have the ability to utilize the higher molecular weight aromatic polycyclics.

## LIFE CYCLE OF PHANEROCHAETE CHRYSOSPORIUM

To adequately harness the striking abilities of the wood rotting fungi, it is necessary to understand their life cycle to benefit the optimization of the treatment process. The life cycle of Hymenomycetes fungi is characterized by many structures formed during vegetative, sexual, and asexual reproductive phases. The thallus is the basic vegetative body of the fungus (9).

Growth occurs in all directions rather than from an apical point. The thallus mainly functions to assist the absorption, assimilation, and accumulation of food. Reproductive bodies develop using the thallus as a base and the thallus plays a reproductive role by becoming a reproductive structure itself.

The fungal mycelium is a mass of interwoven filamentous hyphae usually submerged in growth medium. The mycellium passes through three distinct stages of development. The vegetative phase is the longest and dominant growth phase. The highest concentration of extracellular enzymes are secreted during the vegetative phase. Eventually the tissues of the tertiary mycelium differentiate into fruiting bodies that are shed depending on environmental conditions. Asexual reproduction can occur anytime during the vegetative growth phase. P. chrysosporium produces asexual spores prolifically and at all stages of the life cycle (16).

## ENZYME ACTIVITY STUDIES

It has been shown that P. chrysosporium produces at least ten extracellular hemoproteins and roughly half have ligninase activity (17). The enzyme component designated H8 has been used by several researchers to characterize the ligninase activity. Depending on culture conditions, H8 can be displaced as the major component in favor of H2. The extracellular hemoproteins have distinct amino acid sequences hence they are separate gene products and not merely degradation products of a single precursor. The heme components H3-H5 have manganese peroxidase activity. This three component fraction catalyzes a hydrogen peroxide-dependent oxidation of Mn(II) to Mn(III) but lacks the specificity of H8 to cleave the alkyl sidechains. Two reports (18,19) of soluble manganese ion acceleration on this fraction

prompted further inspection of the enzymatic activity of these peroxidases. The current status of this research is unclear with respect to the reported accelerations, spectral contamination has been observed that contributes to a complex situation for rate data interpretation (20).

The heterogeneity of the various extracellular proteins produced by P. chrysosporium points to possible functional differences among them important to pollutant degradation. Presently there are attempts to uncover substrate specificities where information indicates that they may exist. Oxidation of aromatic substrates by peroxidases leads to the formation of cation radicals that may be sufficiently stable to diffuse some distance from the active site of the enzyme. These cation radical intermediates can be viewed as possible "oxidant" intermediaries leading to the oxidation of other substrates at sites remote to the enzyme and fungal hyphae.

The ability of the ligninase H8 to oxidize polynuclear hydrocarbons has been related to the ionization potential of these compounds. When pyrene is used as a substrate with H8 both pyrene-1,6-dione and pyrene-1,8-dione are the major products. In similar fashion, anthracene is converted to anthraquinone and benz[a]anthracene yields 7,12-benz[a]anthraquinone (14). Both the pyrene-1,6-dione and pyrene-1,8-dione are mutagenic by the Ames test. When the compounds are presented as substrates to the fungus these diones do not accumulate.

Dibenzodioxin and 2-chlorodibenzodioxin are oxidized by the H8 ligninase in the presence of hydrogen peroxide (14). Only determination of radical cation intermediates by flow cell ESR studies was made for these compounds without any product identification. Current work is directed to determine whether chlorinated aromatics are substrates (21,22). Chlorinated phenols have been found to be very suitable substrates, and product identification is under way (13). The individual ligninases have been assayed for their ability to oxidize 2,4,6-trichlorophenol. The activities determined in this process are in order of magnitude lower than those for the degradation of lignin. Considering, the chemical processes involved, this is not too surprising. In the case of one extracellular enzyme, lignin degrading activity was found to inadequately predict the ability to oxidize 2,4,6-trichlorophenol.

## DETOXIFICATION TECHNOLOGY DEVELOPMENTS

### Water Treatment

A water treatment process (MyCoR - Mycelial Color Removal) using P. chrysosporium is under investigation at the bench and

is slated for scale up (23,24). Based on the organism's ability to degrade polynuclear aromatic compounds especially the multiring compounds that may be degraded by bacterial species slowly, the first application of this technology will be the treatment of waste derived from wood treating waste sites. The patented reactor (25) is a specially designed rotating biological contactor that utilizes P. chrysosporium as the biological species for treatment. Optimal growth conditions in the reactor for the fungus are 40°C, a pH of 4.5 and a 100 percent oxygen atmosphere. Since the fungus does not have the same means to adhere to a surface as do bacterial species, the reactor design is modified to permit attachment of the mycelial mass to the plates. This reactor has been used to treat pretreated gasification wastewater for simple color removal with hydraulic retention times of one day, showing 32% color removal for raw wastewater (26). Color removal was found to be dependent on initial color concentrations and active fungal decolorization lifetimes. Recent results derived from the bench scale operation of this technology show that this reactor will degrade 250 ppm pentachlorophenol in water to 5 ppm in 8 hr (27). Pink water associated with munitions production is adequately treated. Degradation of 2,4,6-trinitrotoluene and 2,4-dinitrotoluene in concentrations up to 150 ppm occurs in 24 hr (27). In both cases several sequential doses of the original concentration of contaminant are removed to the same extent. These results indicate that metabolism of substrate is occurring and not merely absorption onto the mycelial mat. Plans to scale up this technology in the next twelve months include pilot scale operations to treat surrogate waste. Once operational conditions are established leachate derived from an actual wood treating site will be treated. Preliminary designs for this treatment at full scale call for parallel treatment trains.

Soil Treatment (28,29)

The general success of solution biodegradation studies with the fungus stimulated speculation that this microorganism may be an appropriate candidate for the treatment of contaminated soils. Attempts to innoculate environmental matrices with nonnative microorganisms have met with varying degrees of success (30). The elucidation of optimal practices leading to successful innoculation of contaminated environmental materials remains to be discovered. At the outset of this research, P. chrysosporium was not known to inhabit the soil. Due to this general lack of knowledge of the habitat, a rather cautious research effort was engaged to determine the ability of the fungus to inhabit and thrive in the soil. Recent research has

assessed the effects of selected soil types, temperatures, pH, and water potentials on the growth of the fungus in sterile and non-sterile soils. Three well characterized soils (two topsoils and a subsoil) were used in this work. Biomass accumulations as well as growth habit of P. chrysosporium were greatly influenced by soil type. Soil nitrogen content appears to be the primary factor responsible for differences in fungal growth in the three studied soils. Growth was strongly and positively correlated with nitrogen content. This factor, therefore, appears to play a major role in mediating the growth of the fungus in the soil, and is easily controlled by nitrogen supplementation.

Increasing the soil water potential from -1.5 MPa to 0.03 MPa resulted in greatly increased growth of P. chrysosporium. Research data suggest that fungal growth might benefit from soil water potentials greater than -0.03 MPa. Water potential is another easily controlled soil factor (31).

Early work indicated that P. chrysosporium did not grow well in non-sterile soils; this may be attributable in part due to ineffective competition with the indigenous microflora. These results were anticipated since the soil is not the normal habitat of P. chrysosporium. Lately, it has been found that growth within the soil can be accomplished through the use of larger quantities of inoculum.

The white rot fungus grows over a wide range of temperatures. Growth has been assessed from 10-39°C. No growth was observed at 10°C whereas growth significantly increases with temperature from 15 to 30°C. No significant difference in growth was recorded between 30 to 39°C. Soil temperatures under field conditions can be controlled by selecting the normal warm months and by soil solarization.

The initial application of the fungus to soil treatment is the remediation of wood treating sites. Target pollutants identified for treatment at these sites are pentachlorophenol and the major aromatic hydrocarbon contaminants found in creosote (napthalene, anthracene, and phenanthrene). Creosote has been extensively characterized and minor constituents of creosote will be added to the dosing mixture as the work progresses when deemed necessary (32). The degradative ability of the fungus in the soil will be evaluated through the measurement of evolved carbon dioxide and material balances will be derived by the measurement of parent compound disappearance and the determination of identity and quantity of metabolic intermediates.

Initial experiments monitoring the mineralization of [U-$^{14}$C] pentachlorophenol in the three soils was somewhat disappointing. Roughly 5% conversion to carbon dioxide was observed observed for the fungus in soil cultures at 39°C and under an atmosphere of 100% oxygen. This low conversion is to

be contrasted with liquid culture mineralization of 50%. Clearly, the degradation in the soil is more complex than we first expected. We are currently gaining a more complete knowledge of the metabolite chemistry of pentachlorophenol and how this chemistry is modified in the soil. There are a variety of reasonable explanations for the observed behavior of the fungus in the soil. We reserve further discussion until sufficient supplemental information is gathered to clarify the overall picture. In conclusion, this fungal system has several features that continue to support its use as a degrader of recalcitrant xenobiotics found in contaminated soil. These abilities include the following: 1) the lignin degrading system of the fungus has a broad substrate specificity, 2) the degrading ability is induced by nitrogen starvation and, 3) the rate and extent of degradation is dependent on the amount of carbohydrates available to the fungus for energy production. Assessment of the amounts and fates of residual pentachlorophenol and its biotransformed products will lead to a better understanding of the degradative ability of P. chrysosporium leading to oxidation of the phenol to carbon dioxide or incorporation into soil organic constituents.

Future research in the soil application will include small scale treatment of selected pollutants at environmentally significant concentrations, the evaluation of amendments on primary and secondary metabolism, and the delivery of oxygen within the soil to the growing fungus. Ancillary investigations will include the development of analytical procedures to assay the fungal growth within the soil, the importance of soil sterilization to growth of the fungus, and inoculum development.

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